1802

PATENT
Attorney Docket: 22671

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Britta HARDY et al.

Serial No. 08/380,857

Filed: January 30, 1995

For: IMMUNO-STIMULATORY MONOCLONAL ANTIBODIES

TRANSMITTAL LETTER

Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

Submitted herewith for filing in the U.S. Patent and Trademark Office are the following:

1) Submission of Foreign Application from which Priority is Claimed under 35 U.S.C. § 119; and
2) certified copy of prior Israel Patent Application No. 108501.

Please charge any fee deficiency, or credit any overpayment, in connection with this matter to Deposit Account No. 14-0112. A duplicate copy of this form is enclosed.

Respectfully submitted,

NATH, AMBERLY & ASSOCIATES

Date: May 23, 1995

Gary M. Nath
Reg. No. 26,965

NATH, AMBERLY & ASSOCIATES
1835 K Street, N.W.
Suite 750
Washington, D.C. 20006
Tel: (202) 775-8383
Fax: (202) 775-8396
smc22671/tl

PATENT
Attorney Docket: 22671

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6/30/95
G.E.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Britta HARDY et al.

Serial No. 08/380,857

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For: IMMUNO-STIMULATORY MONOCLONAL ANTIBODIES

SUBMISSION OF FOREIGN APPLICATIONS FROM WHICH PRIORITIES ARE CLAIMED UNDER 35 U.S.C. § 119

Commissioner of Patents and Trademarks
Washington, D. C. 20231

Sir:

In the matter of the captioned application, applicants submit herewith a certified copy of Israel Patent Application No. 108501 filed January 31, 1994, from which priority is claimed under 35 U.S.C. § 119.

Respectfully submitted,

NATH, AMBERLY & ASSOCIATES

Date: May 23, 1995

Gary M. Nath
Reg. No. 26,965

NATH, AMBERLY & ASSOCIATES
1835 K Street, N.W.
Suite 750
Washington, D.C. 20006
Tel: (202) 775-8383
Fax: (202) 775-8396
smc22671/priority

STATE OF ISRAEL

This is to certify that annexed hereto is a true copy of the documents as originally deposited with the patent application particulars of which are specified on the first page of the annex.

זאת לתעודה כי רצופים בזה העתקים נכונים של המסמכים שהופקדו לכתחילה עם הבקשה לפטנט לפי הפרטים הרשומים בעמוד הראשון של חנספח.

This 20-03-1995 היום

רשם הפטנטים
Registrar of Patents

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108501 מספר: Number

31-01-1994 תאריך: Date

הוקדם/נדחה
Ante/Post-dated

PATENT LAW, 5727 - 1967

בקשה לפטנט
Application for Patent

אני, (שם המבקש, מענו ולגבי גוף מאוגד - מקום התאגדותו)
I (Name and address of applicant, and in case of body corporate-place of incorporation)

Kupat-Holim Health Insurance Institution of the General Federation of Labour in Eretz-Israel, an Ottoman Society, of 101, Arlozorov Street, Tel-Aviv.

פת חולים של ההסתדרות הכללית של העובדים בארץ ישראל, אגודה עותומנית, תובתה, רח' ארלוזורוב 101, תל - אביב.

בעל אמצאה מכח ..... שמסה הוא
Owner, by virtue of ..... of an invention the title of which is

assignment by the inventors

1) Britta Hardy 2) Abraham Novogrodsky

(בעברית) (Hebrew) 1. בריטה הרדי 2. אברהם נובוגרודסקי

(באנגלית) (English)

NOVEL ANTIBODIES AND PHARMACEUTICAL COMPOSITIONS CONTAINING THEM

נוגדנים חדשים ותכשירים תרופתיים המכילים אותם.

מבקש בזאת כי ינתן לי עלית פטנט
hereby apply for a patent to be granted to me in respect thereof.

• בקשת חלוקה — Application of Division	• בקשת פטנט מוסף — Application for Patent Addition	• דרישה דין קדימה Priority Claim		
מבקשת פטנט from Application	• לבקשה/לפטנט to Patent/Appl.	מספר/סימן Number/Mark	תאריך Date	מדינת האגוד Convention Country
No. ..... מס'	No. ..... מס'			
dated ..... מיום	dated ..... מיום			
• יפוי כח: כללי / מיוחד — רצוף בזה / עוד יוגש P.O.A.: general/individual-attached/to be filed later-				
filed in case ..... הוגש בענין				

המען למסירת מסמכים בישראל
Address for Service in Israel

Cohen Zedek & Rapaport
P.O.Box 33116, Tel-Aviv, Israel
63351 /94

חתימת המבקש
Signature of Applicant

COHEN ZEDEK & RAPAPORT

By:

היום ..... 20th ..... בחודש .. January .. שנת ..... 1994 .....
This ..... of the year .....

לשימוש הלשכה
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טופס זה, כשהוא מוטבע בחותם לשכת הפטנטים ומושלם בספר ובתאריך ההגשה, הנו אישור להגשת הבקשה שיפרטיה רשומים לעיל.
This from, impressed with the Seal of the Patent Office and indicating the number and date of filing, certifies the filing of the application the particulars of which are set our above.

• מחק את המיותר Delete whatever is inapplicable

NOVEL ANTIBODIES AND PHARMACEUTICAL COMPOSITIONS CONTAINING THEM

נוגדנים חדשים ותכשירים תרופתיים המכילים אותם

Field of the invention

There are a variety of biological compounds collectively called biological response modifiers that stimulate the immune response and destruct tumor cells. The active ingredients of this invention are novel monoclonal antibodies, that stimulate the immune system and exert a profound therapeutic effect against a variety of tumors.

Introduction

Cancer remains a major cause of death in humans. One third of all individuals in the United States will develop cancer and 20% of Americans die from cancer (494,000 in 1988).

In recent years, in addition to surgery, radiation and chemotherapy a new therapeutic modality has been introduced that has made use of biological response modifiers (BRMs). Immunotherapy is included in this modality and consists of treatment with cytokines, activated mononuclear cells (lymphokine activated killer cells) and antibodies. The most widely studied BRMs are Interleukin-2 and interferon- .
Their mechanism of action include direct effect on the tumors, enhancement of non-specific killing mechanisms (e.g. stimulation of natural killer cells) and augmentation of immunological specific

mechanisms. The clinical use of BRMs resulted in limited success, in part because of the toxicity of these agents. Active immunization against human cancer was also ineffective in most of clinical trials. Hybridoma technology is being used today for the production of monoclonal antibodies. Monoclonal antibodies both labeled and unlabeled were evaluated for therapy and diagnosis. However, no monoclonal antibody has yet become part of the standard care for patients with cancer. T lymphocytes produce and regulate cell-mediated immunity including allograft rejection, delayed cutaneous hypersensitivity and anti-tumor effects.

Agonistic monoclonal antibodies (mAbs)to a number of T cell determinants were previously found to induce proliferation, activation or differentiation of T cells. The best known of such mAbs is the one directed against the CD3 antigen. Triggering of the CD3/TCR complex results in T cell proliferation mediated through interleukin-2 (IL-2) receptor expression and IL-2 production. mAbs to CD3 determinants under certain conditions were able to induce clonal proliferation, to elicit mitogenic activity and also to trigger the cytolytic process in T lymphocytes. In vivo anti-tumor activity of anti CD3 was also described. An alternative pathway of triggering of T cells involves receptor CD2 antigen. As for CD3, effective stimulation via CD2 triggered also the cytolytic process in T lymphocytes. Recently it was found that stimulation of T cells via CD2 and TCR/CD3 resulted in synergistic activation.

Additional T lymphocyte agonistic mAbs were also reported . Activation of helper T lymphocytes was found by mAb 5/9 on a subset of resting T cells. Anti IF7, directed against an epitope of CD26 elicited a co-mitogenic response in human CD4+ lymphocytes. Several other distinct T cell surface antigen may also be capable of eliciting human T cell activation. These include CD28, CD26 and Tal antigens.

The present invention relates to immune-stimulatory monoclonal antibodies for the treatment of cancer, AIDS, and a variety of immune-deficient and immune suppressed conditions.

Generally, the unit dosage form contains from about 0.05mg/kg to 5mg/kg antibody administered intravenously. These monoclonal antibodies were prepared against membranes from a human B lymphoblastoid line (Daudi) using hybridoma technology. These novel mAbs bind to a membrane determinant that differs from those described previously for other agonistic mAbs. The active substance can be administered in lyposome form.

A representative hybridoma according to the present invention was deposited at CNCM, Collection Nationale de Cultures de Microorganismes, Institute Pasteur, 25, Rue du Docteur Roux, 75724 Paris Cedex 15, under Deposit number Bat-1 I-1397, on January 28th, 1994.

Material and Methods

Production of monoclonal antibodies

Balb/c mice were immunized with membrane preparation of Daudi cells. Membranes from Daudi cells were prepared by the glycerol load-hypotonic shock method (Jett, M. Seed, T.M. and Jamieson G.A., J. Biol Chem. 252, 2134, 1977). $50\text{-}80 \times 10^6$ cells suspended in PBS and incubated at 37°C were gradually loaded with 30% glycerol. After 5 min incubation on ice, they were centrifuged and resuspended in cold Tris lysate buffer (containing 10mM Tris-HCl, 1mM $MgCl_2$, 1mM $CaCl_2$, pH 7.4) mixed for 5 min at 4°C and centrifuged at 700g. Supernatants were removed and centrifuged at 3300g (10 min 4°C). The pellet was washed again and the two supernatants containing membrane fraction were pooled. 260µl of membrane preparation (3mg/ml) were emulsified with 260µl of complete Freund adjuvant and injected i.p. into Balb/c mice. Three weeks later the spleens of mice were removed. Splenocytes

were fused with the myeloma cell line NS-0 at a ratio of 10:1. Fusion was performed using polyethylene glycol and the hybridomas were grown in selective media according to Kohler and Milstein (Kohler, G., & Milstein, C., Nature (London) 256, 495, 1975).

A cell bound enzyme linked immunosorbent assay (ELISA) was used to screen supernatants from growing hybridomas (Glassy MC & Surh CD, (1985) J. Immunol. Method. 81 115) which bind to Daudi cells. Positive hybridoma supernatants were then further selected by their ability to induce proliferation of human PBM using [3H] thymidine uptake assay. Positive clones were subcloned by limited dilution, repeatedly tested, expanded and grown in culture.

MAbs were purified from culture medium by 50% ammonium sulfate preciptiation, followed by extensive dialysis against PBS . Further purification was performed by affinity chromatography on sepharose bound anti-mouse antibody columns.

Culture medium

All cells were suspended in RPMI 1640 medium supplemented with 10 percent fetal calf serum (FCS), Na-pyruvate (1.1 mg/ml), L-glutamine (0.3 mg/ml) and antibiotics (Penicillin 200 u/ml and streptomycin 10μg/ml) and incubated in 5% CO_2 humid incubator.

IL-2 units used are Cetus units (1 Cetus unit equals 3 International units).

Cell preparations

Human peripheral blood mononuclear cells (PBM) were obtained from healthy adult donors by ficoll-hypaque density centrifugation (Histopaque, Sigma). PBM were depleted of monocytes by sephadex G10 columns. T cells were separated by SRBC rosetting method. Depletion of CD3 positive and Leu19 positive cells was performed by

immunomagnetic technique. Cultures of PBM incubated for 5-6 days with BAT mAbs or control were washed three times with PBS. Unconjugated antibodies to either CD3 or Leu19 (CD56) were added to the cells in complete RPMI medium and incubated for one hour at 4°C. Magnetic beads coated with antimouse antibody were added for 30 min. The cells attached to the beads were removed with the magnet and the unattached cells were analysed for cytotoxic activity and stained by flow cytometry.

Cytotoxicity assay

Cytotoxicity assays were done as follows: $2\text{-}4x10^6$ target cells were mixed with 200μCi of ^{51}Cr-chromate for 1h in serum free medium. They were washed three times with complete medium and finally resuspended in RPMI-10% FCS and plated at 10^4 cells per well. Effector cells were cultured lymphocytes prepared from normal peripheral blood incubated for various time periods with the different mAbs, isotypic control IgG or with IL-2. Prior to the assay the cells where washed three times in RPMI medium, stained for viability cells using 1% Trypan blue, mixed with target cells at various effector-target ratios in round bottom microtiter plates and incubated for 3h at 37°C in a 5% CO_2. The culture supernatants were harvested and counted in a γ-scintillation counter. Maximum isotope release (MR) is produced by incubation of the target cells with triton x-100. Spontaneous release (SR) is measured by incubation of the targets with medium alone. Percentage of cell lysis is caclulated by (ER-SR/MR-SR)x100, where ER is the experimental effector release.

Induction of cytotoxity in human lymphocyte subpopulations.

PBM cells (4.10^6/ml) were cultured for 6 days in the presence of BAT mABs. Thereafter the cells were washed three times and were depleted

CD3 and Leu19 cells by magnetic beads coated with anti-mouse F(ab')2 and tested for cytotoxicity against K562 and Daudi cells.

Flow cytometry

Cell surface antigens were detected by flow cytometry using a FACS 440 (Becton-Dickinson). For each analysis 10^6 cells were used. The cells were stained by sequential incubation with an optimal concentration of murine mAb to human CD3, IL-2 receptor, or the mAb BAT 1-9 that we have produced. FITC-conjugated goat anti-mouse $F(ab')_2$ was used as a second antibody in this indirect staining. Each incubation was carried in PBS pH 7.4 containing 1% BSA and 0.5% Na-azide for 30 min at 4°C and was followed by three washings with the same buffer. 10^4 stained cells were analysed.

Detection of BAT mAb binding determinant(s).

Detection of BAT mAb binding determinant(s) on human peripheral T cell, Jurkat T cell line and Daudi B lymphoblastoid cells was done using blott Western technique. Briefly, $50x10^6$ cells/ml suspended in PBS were gradually loaded with 30% glycerol and membranes were separated by sequential centrifugations.

Samples of the membrane preparations were separated by SDS-PAGE and then transferred to nitrocellulose blots which were immersed in 1% low fat milk in PBS. Detection of the BAT mAb binding protein in the nitrocellulose blots was done by incubating the blots with BAT mAb for 2h at room temperature, followed by 30 min incubation with peroxidase conjugated goat anti-mouse antibody. Detection of bands was done using ECL.

Mouse tumor models.

Three mouse tumor models were used: B16 melanoma, Lewis lung carcinoma (3LL) and methylcholanthrene induced fibrosarcoma (MCA 105).

50-200x10^6 cells were injected i.v. to C57BL mice (8 weeks of age). Two weeks later, BAT-1 was injected (i.v.), 1-10μg/mouse and 10 day later mice were sacrificed and established lung metastases were counted.

RESULTS

Binding characteristics

Nine monoclonal antibodies to B lymphoblastoid cells that were selected for binding to Daudi cells and then for inducing human peripheral blood lymphocytes proliferation, were designated as BAT 1-9 mAbs. The isotypes of BAT mAbs were determined by both ELISA and Ochterlony assays. BAT 1,2,3,6,7 and 9 were found to be of IgG1 class whereas BAT 4 and 5 were of the IgM class. BAT 8 was IgG2a.
We tested their binding to purified peripheral human T cells by indirect immunofluoresence staining. Fig. 1 demonstrates FACS analysis of such an experiment. As can be seen, BAT mAbs bind to peripheral blood T cells. The extent of binding varied from BAT-2 44% BAT-5 38%, BAT-1 32% to a somewhat weaker binding of BAT-4 (13%). Purified peripheral blood B lymphocytes from the same blood donors, did not bind these mAbs (data not shown).
The finding that BAT-1 binds to CD3+ cells was further corroborated using FACS analysis of double labeled cells (Fig. 2).

Binding of BAT mAb to human lymphocyte subpopulations.

Enriched CD3+ and Leu 19+ subpopulations were obtained from human PBM using magnetic beads (see Material and Methods). As can be seen in Table 1, BAT-1 in addition to its binding to CD3 bearing cells binding also to leu19/NK cells.

Binding of BAT mAb to various cell types

Binding of BAT mAbs to various cell types was determined. As can be seen in Table 2 BAT mAbs bind to K562, an erythroleukemia cell line and MCF7, a human mammary carcinoma cell line, in addition to their binding to PBL, Daudi and the Jurkat T cell line. The extent of binding varied among the BAT mAbs and cell types tested. On the other hand, the mouse renal carcinoma (MR28) bound only very little and MEL (murine erytholeukemia) did not bind the BAT mAbs.

Analysis of BAT mAb binding site

In order to determine the molecular weight of the membrane protein that interacts with the BAT-1 mAbs we solubilized membrane preparation of Daudi cells and separated the protein by SDS-PAGE. Nitrocellulose transferred blots were incubated with BAT-1 mAb and bands were detected using LCL. The molecular size of the BAT-1 binding protein was found to be about 48-50 KDa (Fig 3).

We have purified the BAT mAb binding protein using BAT mAb conjugated to Sepharose. This binding protein can also be prepared by cloning using molecular biology techniques. Administration of BAT-1 in vivo could result in the induction of BAT antibodies. This response is associated with in vivo immune stimulation and anti tumor activity.

Functional characteristic of BAT mAbs

BAT mAbs induced thymidine incorporation

Human peripheral blood cells were cultured for 6 days in the presence of increasing concentrations of a panel of BAT mAbs and pulsed with $[^3H]$ thymidine 20 hr prior to harvesting.

As can be seen in Fig. 4 a gradual increase in concentrations of BAT mAbs resulted in a modest but significant increase in thymidine incorporation. High dose of antibody caused a decrease in uptake. We

have performed experiments using isotype matched control antibodies. They were not stimulatory, indicating that the angonistic effect of the BAT mAbs was dependent on their binding specific properties. For example mAb of the IgG1 isotype that was raised in our laboratory against ovarian carcinoma cells failed to show any agonistic effect and this is in contrast to agonistic BAT 1,2,3,6,7 and 9 which also belong to the IgG1 class.

BAT mAbs induce cytotoxicity in human PBM

Cultures of human peripheral blood mononuclear cells incubated with BAT mAbs for various time periods, were tested for their ability to lyse tumor cell lines.

Human PBM incubated for one week with a panel of BAT mAbs rendered cytotoxic for K562, human erythroleukemia (NK sensitive) and RC-29, renal carcinoma cell line (NK resistant) (Table 3).

The kinetics of the increase in cytotoxic activity of human PBM that were stimulated with BAT mAbs was investigated. Maximal cytotoxicity toward human colon carcinoma (HT-29) and renal cell carcinoma (RC-29) was attained after 7 day incubation of human PBM with BAT mAbs (Figure 5).

Characterization of lymphocyte subpopulation involved in BAT-induced cytotoxicity

We have investigated whether the increase in cytotoxic activity of human PBM induced by BAT mAbs, is due to activation of NK cells, T cells or both. For this purpose NK and T cells were purified and their cytotoxicity induced by BAT were determined. For purification we used Leu19 and anti CD3 monoclonal antibodies, followed by anti-mouse IgG coated magnetic beads. This led to depletion of the subpopulations of cells which bind to the corresponding antibody. As

can be seen in Table 4 the number of lytic units increased in both the CD3 depleted and Leu19 depleted cell cultures. BAT 6 and 8 were used in these experiment and the targets were human erythroleukemia (K562) and human lymphoma (Daudi).

Synergism between BAT mAb and IL-2 in the induction of cytotoxicity.

Induction of cytotoxicity in human PBM was studied upon incubation with BAT mAb in combination with IL-2. IL-2 at suboptimal concentrations (1U/ml) was added together with increasing concentrations of BAT-2 mAb. Cytotoxicity was tested after one week in culture against K562 and HT29 tumor cell lines. As shown in Table 5, low concentration of BAT-2 synergized with IL-2 in the induction of cytotoxicity to both targets.

INF-α was previously shown to enhance the expression of MHC-I class antigens. Therefore administration of INF-α is likely to potentiate the anti-tumor effect of BAT which is mediated by cytotoxic cells directed against various tumor cells (Bearing MHC class I antigens). IL-1 and IL-6 which provide accessory signals for T cell activation will also potentiate BAT immune stimulatory and anti-tumor activity.

Immune stimulatory effects of BAT-1 in mice:

In vitro studies

The mAb BAT-1 demonstrates stimulatory properties in murine splenocytes similar to those seen in human PBL. They include:

a. Increased splenocyte proliferation in vitro as measured by ^{3}H thymidine incorporation (Table 6).
b. Synergistic stimulatory effect by incubation of splenocytes with a combination of BAT-1 and IL-2 (Table 6).
c. Increased cytotoxicity in murine splenocytes cultured in the presence of BAT-1 and further increase in cytotoxicity upon

incubation in the presence of IL-2 (Table 7).

Murine tumor target cells that were susceptible to the killing effect by BAT-1 activated splenocytes included: B16 melanoma, Lewis lung carcinoma (3LL), fibrosarcoma (MCA 105), renal cell carcinoma (MR 28) and lymphoma (YAC) (Table 8).

In vivo studies

BAT-1 manifested immune stimulatory effects upon administration in vivo. They included:

a. Stimulation of [3H] thymidine incorporation in splenocytes from mice injected with BAT-1 (Table 9).

   Maximal stimulation (10 fold) was attained upon administration of BAT at doses of 10μg/mouse and 0.01μg/mouse, 10 days and 3 days (respectively) prior to [^{3}H] thymidine incorporation assay.

b. Induction of cytotoxicity in splenocytes from mice injected with BAT-1 (Table 10).

   BAT-1 administered at different doses and schedules induce cytotoxicity towards renal cell carcinoma (MR-28) and lymphoma (YAC). Maximal effect was attained upon administration of BAT at a dose of 1 μg/mouse and 0.01 μg/mouse, 10 days and 3 days (respectively) prior to cytotoxicity assay.

Immunotherapeutic effect of BAT-1 against mouse tumors

1. BAT-1 abolishes lung metastases in B16 melanoma-inoculated mice, using an established lung metastases model

   C57BL mice were injected (i.v.) with $50x10^3$ B16 melanoma cells. 24 days later numerous metastases have devloped in the lungs (practically reached confluency) (Figure 6, upper row).

Mice that were injected with BAT-1 (10μg/mouse) two weeks after inoculation with the B16 melanoma were practically free of metastases (Figure 6, lower row).

Figure 7 summarizes the results of six separate experiments done under similar conditions as above.

2. <u>BAT-1 abolishes lung metastases in Lewis lung carcinoma (3LL)-inoculated mice using an established lung metastases model.</u>

   Experimental conditions were similar to those described for B16 melanoma (see above) except that $2x10^5$ 3LL cells were injected.

   Fig. 8, upper row, shows lungs from 3LL-inoculated mice with numerous metastases. Lower row shows lungs from mice that had been inoculated with the tumor cells followed 14 days later by BAT-1 treatment. <u>Table 11</u> presents quantitative data on the effect of BAT-1 treatment on the number of lung metastases and lung weights.

3. BAT abolishes lung metastases in MCA fibrosarcoma (MCA 105)-inoculated mice using an established lung metastases model.

   Experimental conditions were similar to those described for the 3LL Lewis lung carcinoma model.

   <u>Fig 9</u> upper row shows lungs from MCA 105 inoculated mice with numerous metastases. Lower row shows lungs from mice that had been inoculated with the tumor followed by BAT-1 treatment.

4. <u>BAT-1 cures mice bearing B16 melanoma</u>

   Mice inoculated with B16 melanoma as above die wthin 35 days after tumor inoculation.

   Essentially, all mice that were injected with BAT-1 (10μg/mouse) 14 days after tumor inoculation survived over 100 days. (Fig 10).

Table 1

Binding of BAT-1 mAb to human lymphoid subpopulations

	Positive cells (%)	
	Subpopulation of cells enriched for:	
	CD3+	Leu 19+
CD 3	66.4 (100%)	-
Leu 19	-	59.5 (100%)
BAT-1	19.8 (30%)	42.2 (70%)

Table 2

Binding of BAT mAbs to various cell types

Cells	BAT mAb 1	2	4	5	8	9
Human						
Daudi	+++	++	++	+++	±	++ +
Jurkat	+	±	+++	+++	-	++ +
PBL	+++	±	+	+	±	±
K562	+++	+	+	+++	±	++ +
MCF7	±	++	+++	+++	+	++ +
Mouse						
MR28	-	±	+	+	±	±
MEL	-	-	-	-	±	-

Binding was assayed by ELISA and is expressed as + (0.1-0.2 OD), ++ (0.2-0.3 OD) and +++ (over 0.3 OD)

Table 3

BAT mAbs induce cytotoxicity in human PBM

	Specific ^{51}Cr release (%)	
mAb	K562	RC-29
None	11.0*	9.1
BAT-1	25.1	45.4
2	29.0	23.2
3	26.3	32.3
4	30.6	40.7
5	32.3	34.3
6	15.2	13.8
7	17.8	16.7
8	17.8	19.2
IL2 1000u/ml	57.6	36.9

* Percent lysis of target cells using effector:target ratio of 5:1.

Table 4

BAT mAbs induce cytotoxicity in human lymphocyte subpopulations

	Lytic Units			
	Target cells:			
	K562		Daudi	
	effector cells depleted of:			
	CD3	Leu 19	CD3	Leu19
Control	8	4	10	3.8
BAT 6	25	10	20	13
BAT 8	28	12.5	26	20

Table 5

Synergistic effect of BAT-2 mAb and IL2 in the induction of cytotoxicity in human PBL

Specific ^{51}Cr release (%)

BAT (μg/ml)	Target cells: HT29		K562	
	IL-2 (1 U/ml): -	+	-	+
0	5.8±0.1	7.5±0.3	3.7±0.1	6.9±0.4
2	14.8±1.3	37.6±2.0	12.1±0.4	29 6±1.6
4	16.7±0.6	27.3±1.0	13.3±0.6	18.5±1.2
8	22.0±1.0	15.1±0.6	19.9±0.7	12.5±0.4

Table 6

C57BL murine splenocytes incubated for 5 days in vitro with various concentrations of BAT-1 and in combination with interleukin-2 (1u and 10u per ml)

	cpm [^{3}H] Thymidine x 10^3		
BAT-1	IL2		
(μg/ml)	-	1U/ml	10u/ml
-	1.5±0.07	16.0±0.9	67.5± 1.6
10	11.0±0.4	32.1±1.5	241.6±17.1
1	12.9±0.8	35.9±3.3	247.8± 1.9
0.1	18.1±0.9	51.0±7.3	255.1±18.0
0.001	10.5±0.1	34.1±0.5	215.1±20.8
0.0001	2.6±0.1	13.2±0.9	73.3± 5.6

Table 7

Induction of cytotoxicity in C57BL splenocytes cultures for 5 days in vitro in the presence of various BAT-1 concentrations and in combinations with low dose IL-2.

	Specifc ^{51}Cr release (%)[a]		
BAT-1	Interleukin-2		
(ng/ml)	-	1u/ml	10u/ml
-	7.4	10.0	31.1
100	24.9	31.3	52.4
10	17.4	26.7	52.8
1	12.3	23.9	46.8
0.1	12.2	21.7	45.5

[a] B16 melanoma cells were used as a target.
Effector to target cell ratio was 50:1

Table 8

BAT-1 induced cytotoxicity in splenoyctes against mouse tumor cells (In vitro study)

Mouse strain	Tumor target cells	Specific ^{51}Cr release %[a] BAT-1 -	BAT-1 +
C57BL	B16 melanoma	5.4	13.6
	Lewis lung carcinoma (3LL)	11.2	24.0
	Fibrosarcoma (MCA 105)	27.0	45.0
BALB/C	Lymphoma (YAC)	8	12.2
	Renal cell carcinoma (MR28)	0.1	5.2

[a]Splenocytes were cultured, in vitro, for 5 days, in the absence and presence of BAT-1 mAb (1μg/ml).
Cytotoxicity was determined at an effector/target ratio of 60:1.

Table 9

^{3}H Thymidine incorporation in splenocytes from mice injected with BAT-1 mAb

Time of BAT-1 injection		
10 days[a]	3 days[a]	
Dose of BAT-1 (μg/mouse)		[3H] Thymidine incorporation (cpm x 10^{-3} ±SD)
-	-	3.4±01.1
10	10	19.5±1.2
10	0.01	32.7±0.1
10	-	10.0±0.1
1	1	18.9±0.0
1	0.01	18.4±1.1
1	-	12.1±0.5

[a]Mice were injected I.V. with BAT-1 mAb (at different concentrations) at 10 days and 3 days prior to determination of [^{3}H] Thymidine uptake in isolated splenocytes.

Table 10

Cytotoxic activity of splenocytes from Balb/c mice injected with BAT-1

Time of BAT-1 injection		Specific ^{51}Cr release[b] (% ± SD)	
10 days[a]	3 days[a]	Cell Target	
Dose of BAT-1 (μg/mouse)		MR 28	YAC
none	none	8.8±0.07[b]	3.2±0.2
10	10	19.5±0.1	12.7±0.0
10	1	29.0±1.4	15.6±0.9
10	0.1	27.0±1.0	18.0±0.2
10	0.01	30.9±0.0	21.8±0.9
10	-	24.2±0.2	14.0±1.2
1	1	37.0±3.7	24.4±1.2
1	0.1	46.2±7.0	32.2±1.6
1	0.0-1	62.0±4.0	40.2±1.6
1	-	42.0±0.6	22.5±0.6

[a] Mice were injected C.V. with BAT-1 mAb at different concentrations 10 days and 3 days prior to determination of cytotoxicity in isolated splenocytes. E:T ratio was 60:1

[b] Each group contained 3 mice. The cytotoxic assay for each mouse was done in triplicate, and the mean was calculated. Results are expressed as the mean ±SD of 3 individual mice.

Table 11

BAT-1 mAb prevents lung metastases in C57Bl mice inoculated with Lewis lung carcinoma (3LL)

	Lung weight (grams)		
		Tumor inoculated mice	
	Normal mice	BAT-1 treated	
		-	+
1	0.3	1.097	0.347
2	0.3	0.896	0.219
3	0.331	1.147	0.317
4	0.341	1.094	0.344
5	0.329	1.128	0.324
Mean±SD	0.320±0.02	1.072±0.09	0.310±0.04

CLAIMS:

1. Monoclonal immuno-stimulatory antibodies (BAT) inducing human PBM proliferation and activation, obtained by conventional hybridoma technology, by fusion of splenocytes from laboratory animals immunized with membranes of B-lymphoblastoid cells and selected for binding to Daudi cells and for induction of human PBM activation and proliferation.

2. Monoclonal antibodies according to claim 1, raised against membranes of B-lymphoblastoid cells (Daudi), containing a variable region causing activation and proliferation of lymphocytes.

3. Antibodies according to claim 1 or 2, which bind to a protein determinant in Daudi cells and human T-cells.

4. Antibodies according to claim 3, where the molecular weight of the protein is about 50-K-daltons.

5. Antibodies according to any of claims 1 to 4, being the product of humanization of mouse BAT mAbs.

6. Antibodies according to any of claims 1 to 5, of any isotype class.

7. Hybridomas producing monoclonal antibodies according to any of claims 1 to 6, produced by conventional hybridoma technique from splenocytes of a laboratory animal immunized by a B-lymphoblastoid cell line Daudi membrane preparation selection and screening for the required characteristics.

8. A process for the production of immunostimulatory antibodies (BAT monoclonal antibodies), which comprises immunizing a laboratory animal with a membrane preparation from B-lymphoblastoid cell line (Daudi), proceeding according to conventional hybridoma technique, selecting hybridomas binding to Daudi cells and further selecting hybridomas inducing human PBM proliferation and activation.

9. An injectable pharmaceutical composition containing or consisting of an effective quantity of BAT antibodies, claimed in any of claims 1 to 4.

10. A composition according to claim 9, containing in addition a quantity of IL-2 or another cytokine.

11. An injectable pharmaceutical composition according to claim 9 or 10, for use in the treatment of genetic background immunodeficiency diseases.

12. An injectable pharmaceutical composition according to claim 9 or 10, for the treatment of AIDS.

13. A pharmaceutical composition according to claim 9 or 10, for augmenting the immuno-response of the human body.

14. A pharmaceutical composition for the treatment of maligancies by immunotherapy, containing as active ingredient monoclonal antibodies claimed in any of claims 1 to 6, optionally with IL-2 or another cytokine.

15. A pharmaceutical composition according to claim 10, for use in the treatment of carcinoma (including melonoma), sarcoma, leukemia and lymphoma.

16. A pharmaceutical composition of enhanced efficacy, containing BAT mAbs in combination with a tumor vaccine.

17. A pharameutical composition according to claim 10 or 14, where dosage of IL-2 varies between from 3 x 10^6 IU/day 18 x 10^6 IU/day.

18. A pharmaceutical injectable composition according to any of claims 10 to 17, containing a dosage of from 0.05 mg/kg to 5 mg/kg monoclonal antibodies claimed in any of claims 1 to 5.

19. A pharmaceutical composition according to any of claims 9 to 18, in lipsome form.

20. A method for augmenting the immune system of mammals, and especially humans, which comprises administering to a mammal in need thereof an efficient quantity of a monoclonal antibody defined in any of claims 1 to 5.

21. A method for the therapy of a human malignancy, which comprises administering to a person in need thereof an efficient quantity of a composition according to any of claims 9 to 19.

22. A method for the treatment of a human afflicted with a immunodeficiency disease, which comprises administering to a human in need thereof an efficient quantity of a composition defined in any of claims 10 to 19.

23. Monoclonal BAT antibodies, substantially as hereinbefore described and with reference to any of the Examples.

24. Hybridomas producing monoclonal BAT antibodies, substantially as herein described and with reference to the Examples.

25. Pharmaceutical compositions containing BAT monoclonal antibodies claimed in any of claims 1 to 5, substantially as herein described.

COHEN ZEDEK & RAPAPORT
P. O. Box 33116, Tel-Aviv
Attorneys for Applicant
By:

KUPAT - HOLIM

10 SHEETS
SHEET 1

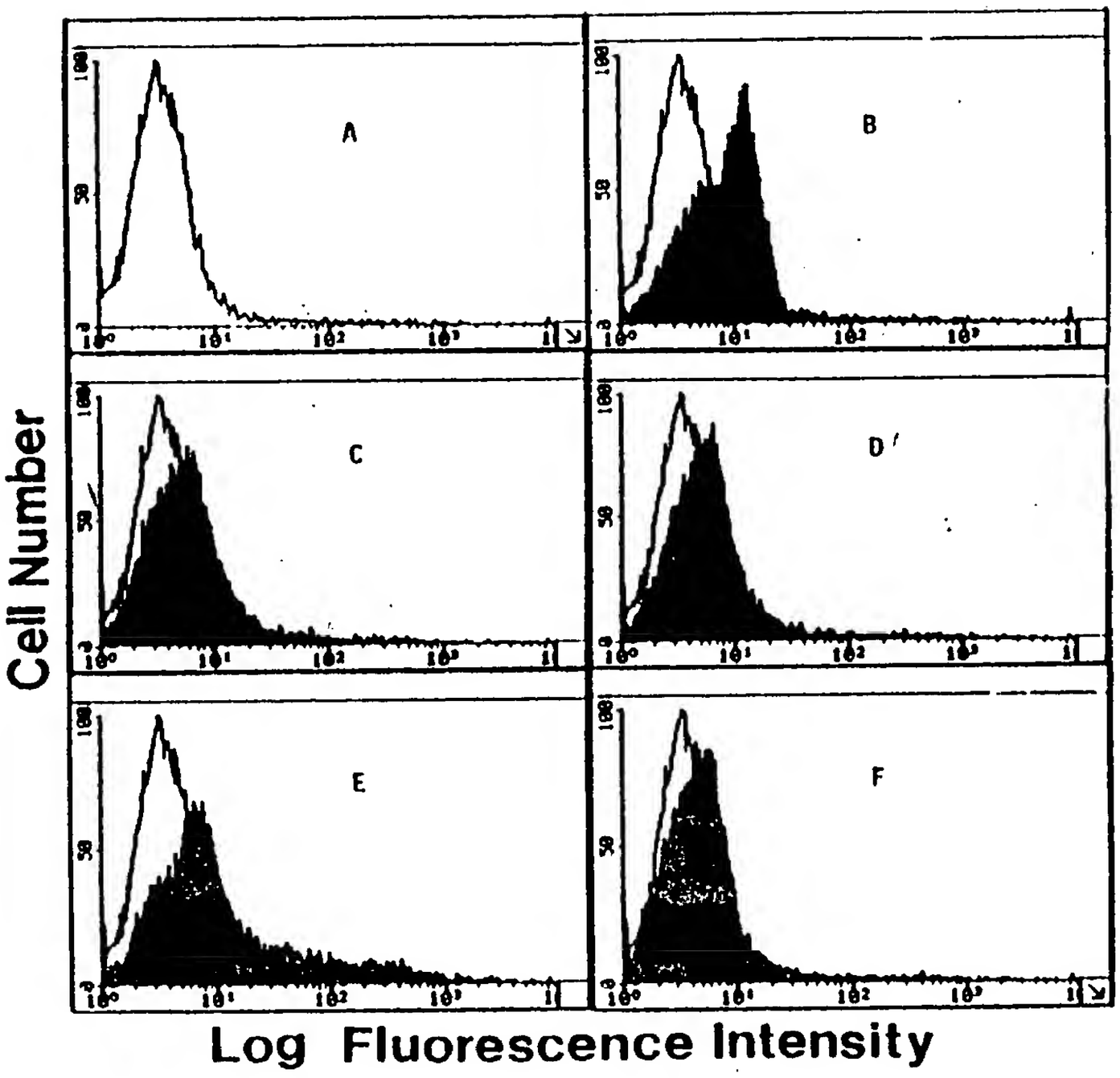


Fig. 1 Binding of BAT mAbs to human purified T lymphocytes: FACS analysis.

Background, no 1st Ab (a); CD3 (b); BAT-1 (c); BAT-5 (D); BAT-2 (E); BAT 4 (f). All were analyzed followed by staining with FITC-goat anti-mouse Ab.

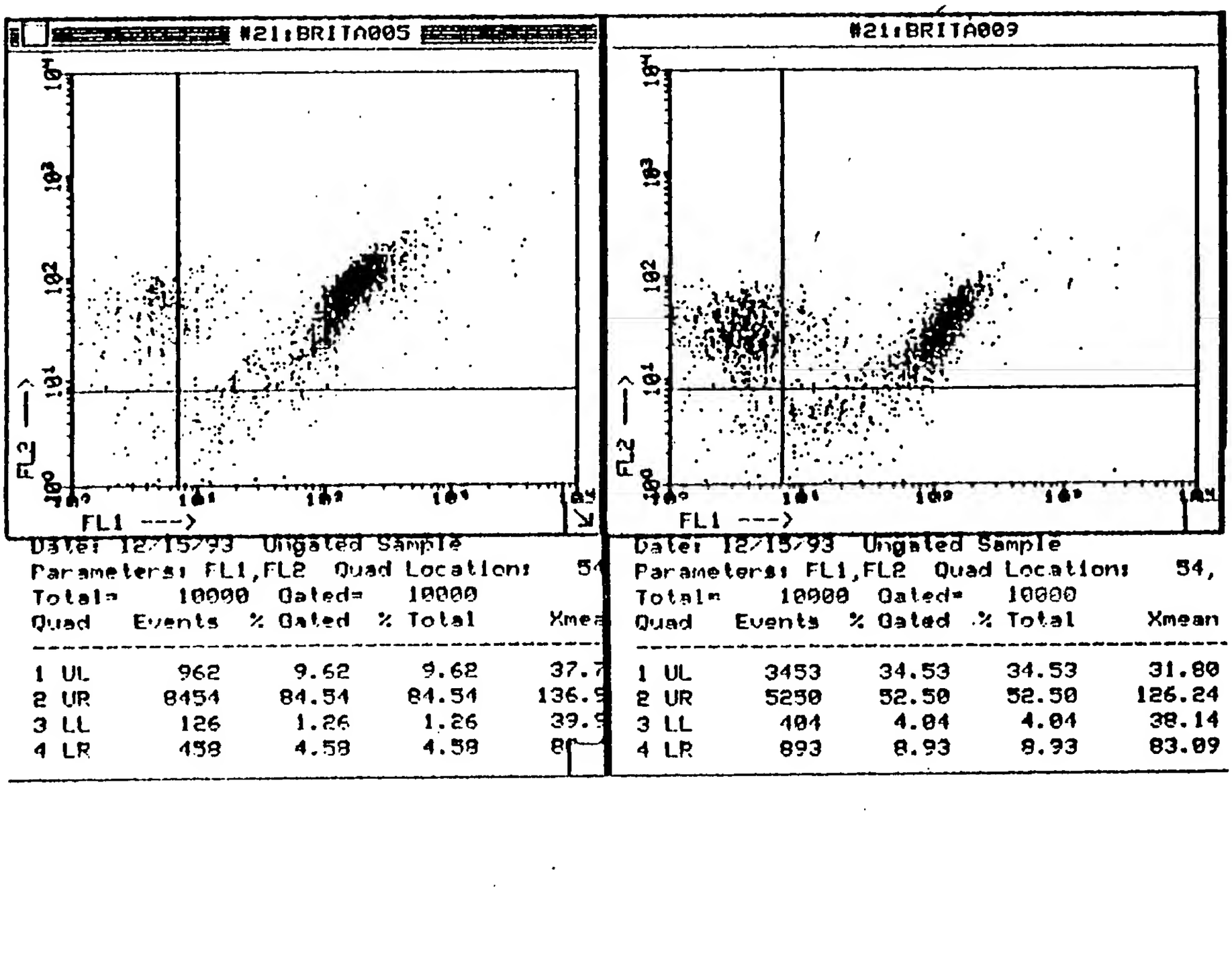
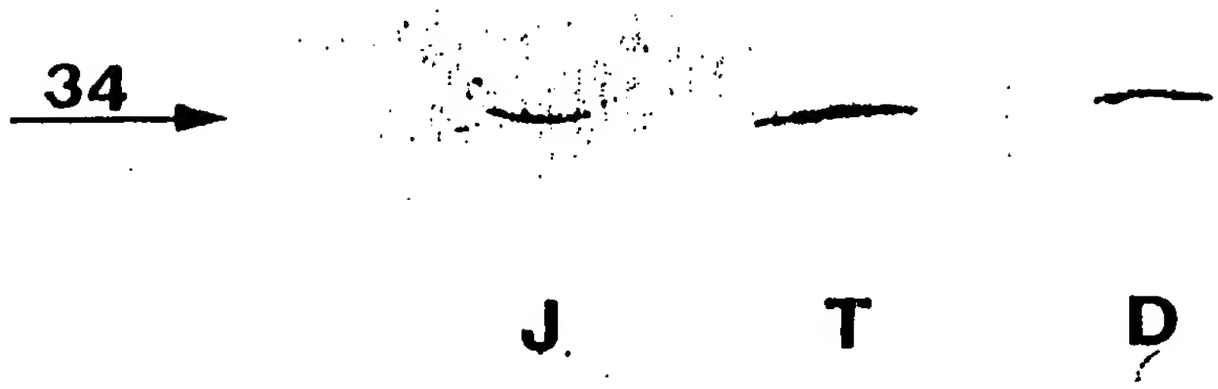


Fig. 2 FACS analysis of human PBM (left) and enriched T lymphocytes (right) double labeled with PE-anti-CD3 and FITC-BAT-1.

Fig. 3 Western blot analysis of the binding of BAT-1 to Daudi (D), human peripheral T cells (T) and Jurkat cell line (J) membrane binding protein.



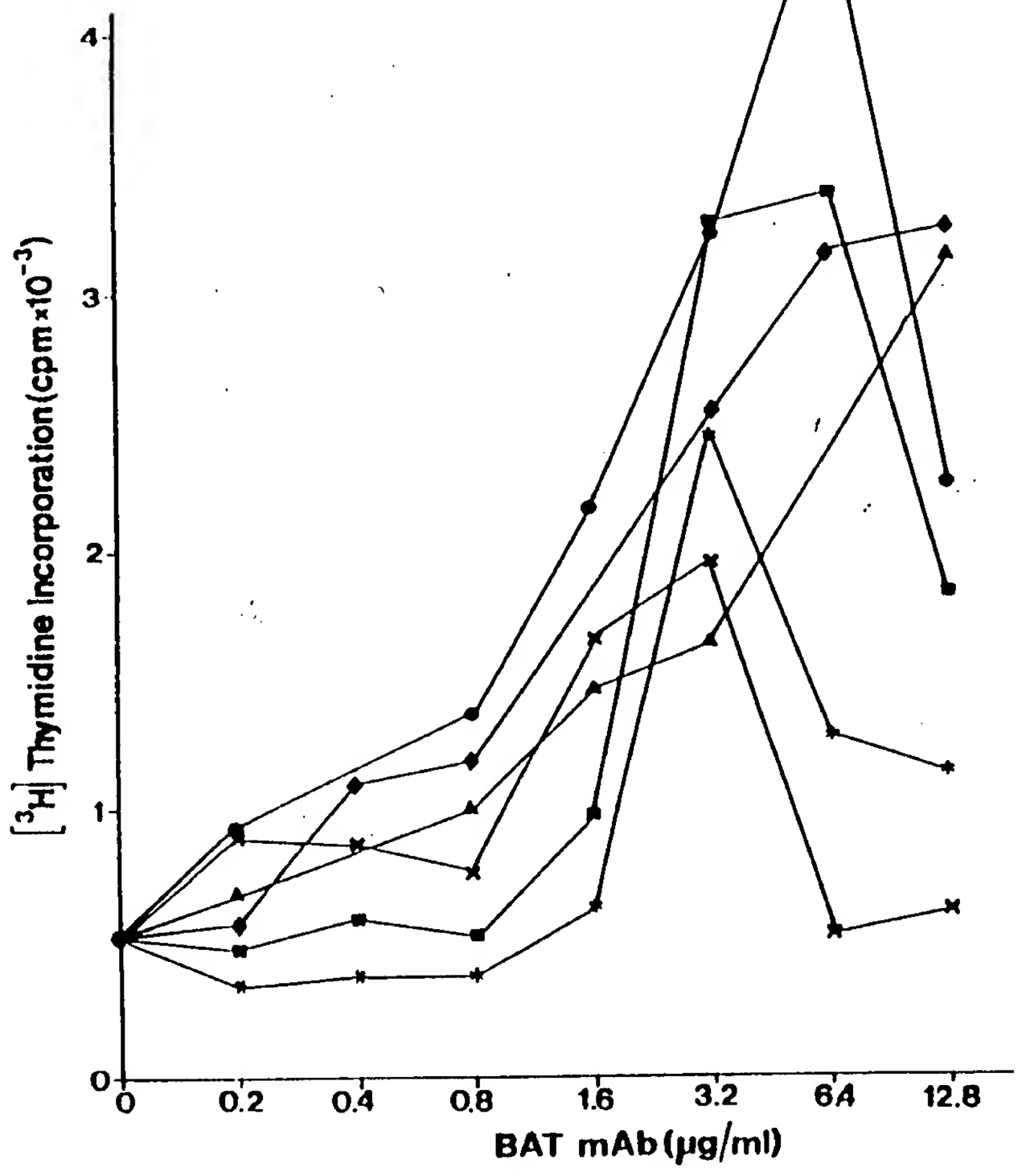


Fig. 4 [^{3}H]Thymidine incorporation in cells cultured for six days in the presence of increasing concentrations of a panel of BAT mAbs BAT-1 (-▲ -), BAT-2 (- X -), BAT-3 (- ● -), BAT-5 (-✳ -), BAT-6 (-◆ -), BAT-7 (-■ -).

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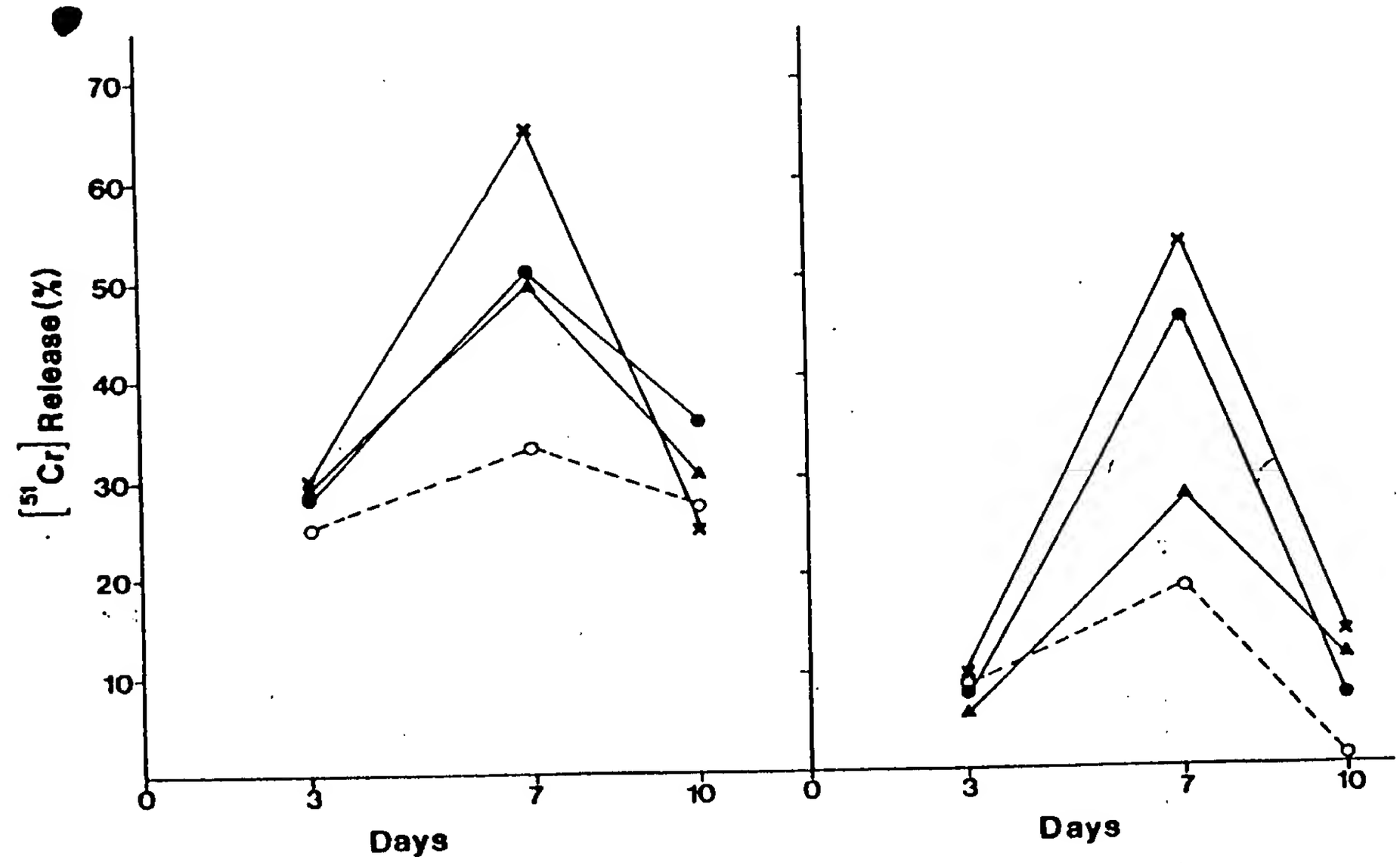


Fig. 5 Induction of cytotoxic activity in PBM cultured for various time intervals with 2.5μg/ml BAT mAbs. HT-29 (left) or RC 29 (right) were used as target cells. Effector to target ratio was 20:1. Control (- O -), BAT-1 (- ● -), BAT-2 (- X -), BAT-3 (-▲-).

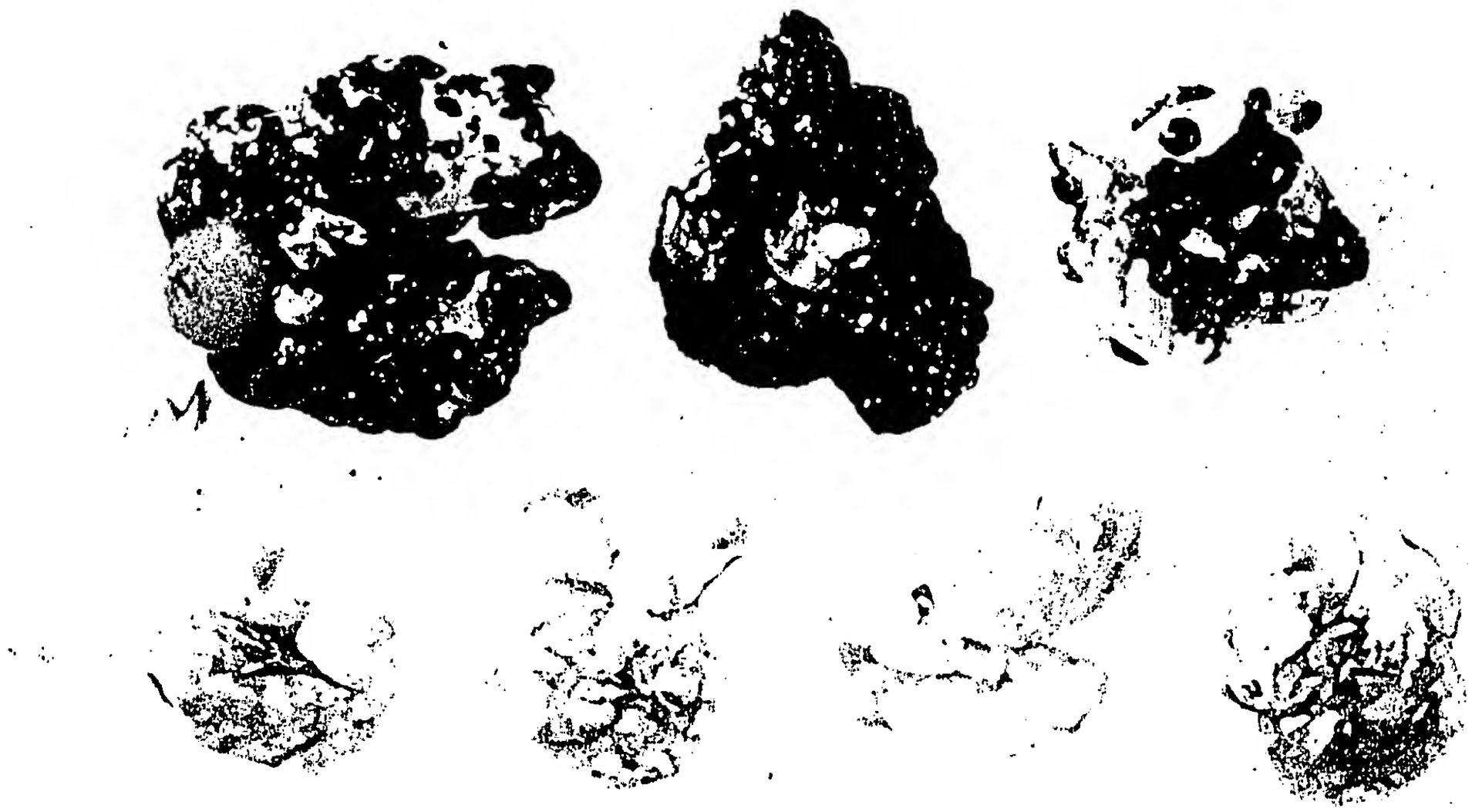
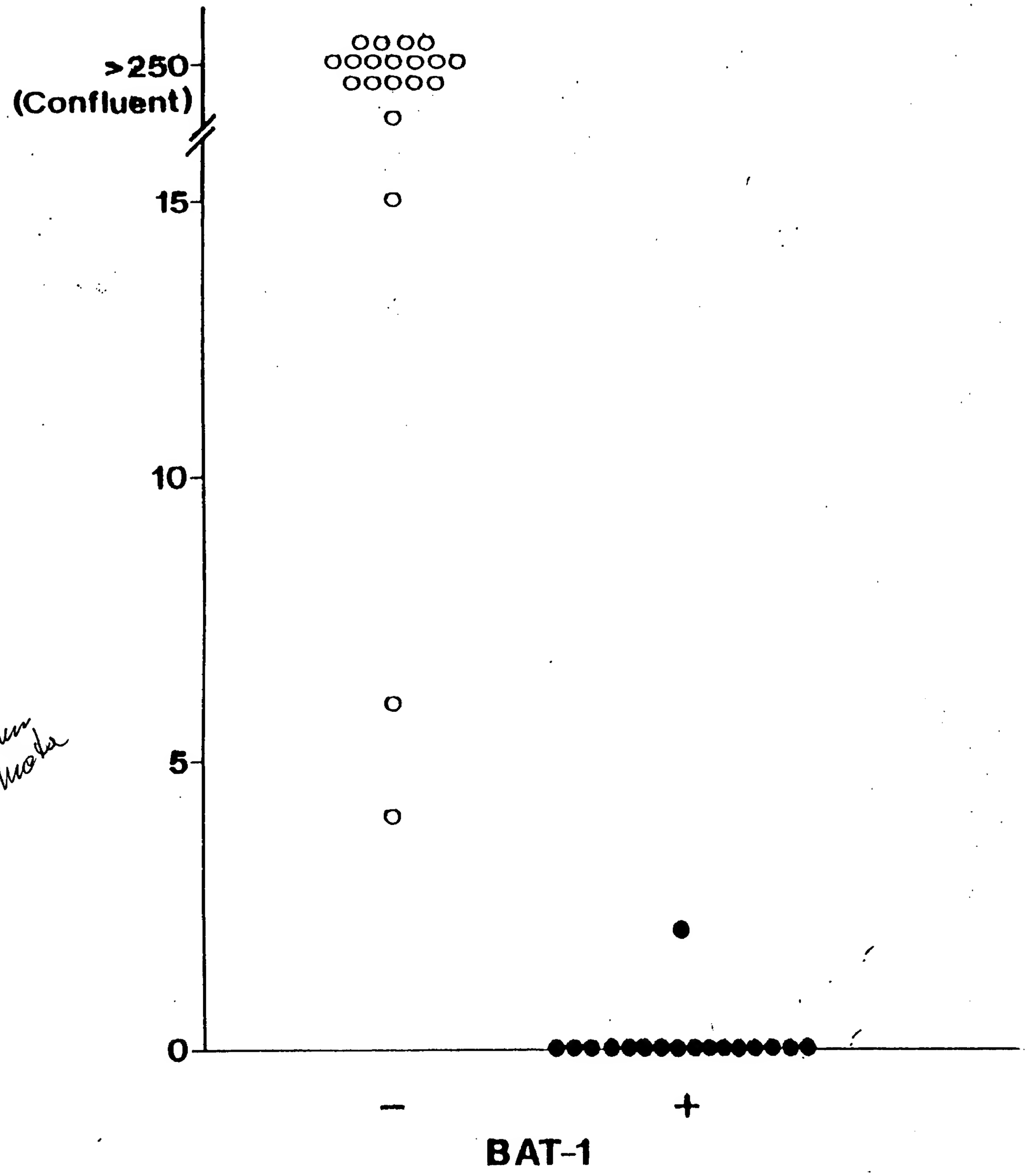


Fig. 6 Effect of BAT-1 on lung metastases in B-16 inoculated mice
Lungs from mice that were inoculated with the tumor alone (upper row), same as above, followed 14 says later by I.V. injection of BAT-1 (10μg/mouse) (Lower row).

Fig. 7 BAT-1 abolishes lung metastases in B-16 melanoma inoculated mice.



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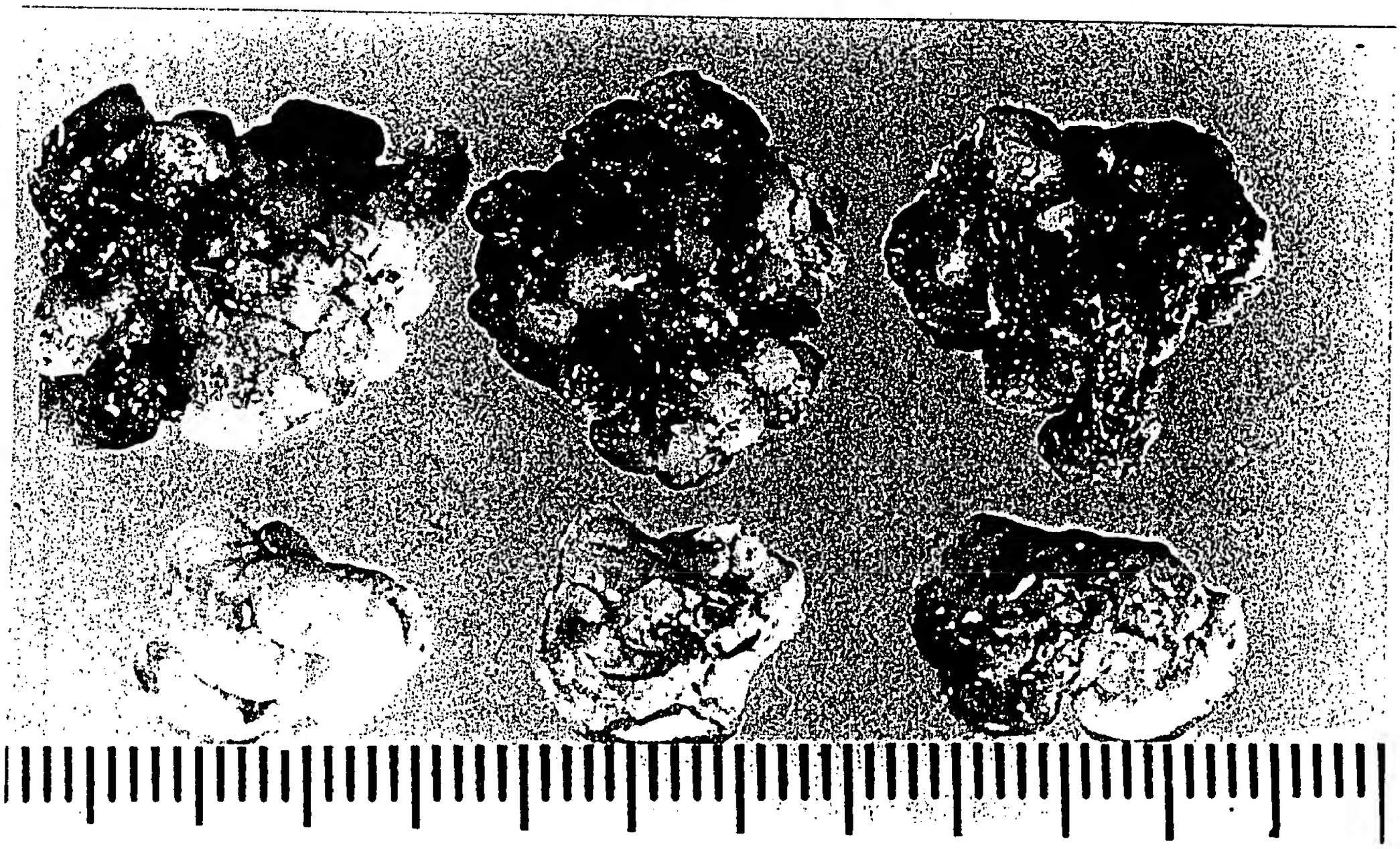

Fig. 8 Effect of BAT-1 on lung metastases in Lewis lung carcinoma-(3LL) inoculated mice

Lungs from mice that were inoculated with the tumor alone (upper row), same as above, followed 14 says later by I.V. injection of BAT-1 (10μg/mouse) (Lower row).

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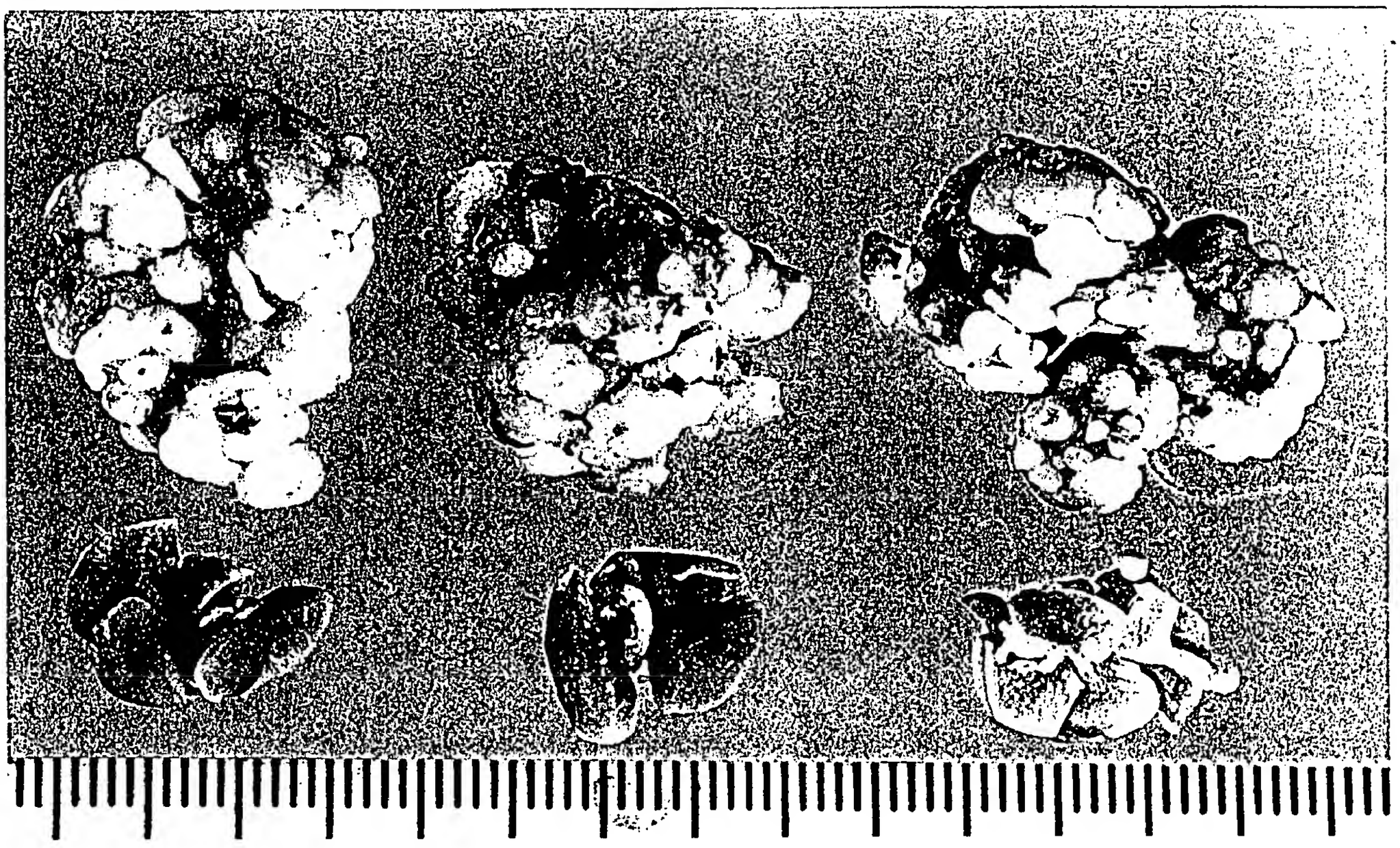

Fig. 9 Effect of BAT-1 on lung metastases in MCA 105 fibrosarcoma inoculated mice

Lungs from mice that were inoculated with the tumor alone (upper row), same as above, followed 14 says later by I.V. injection of BAT-1 (10μg/mouse) (Lower row).

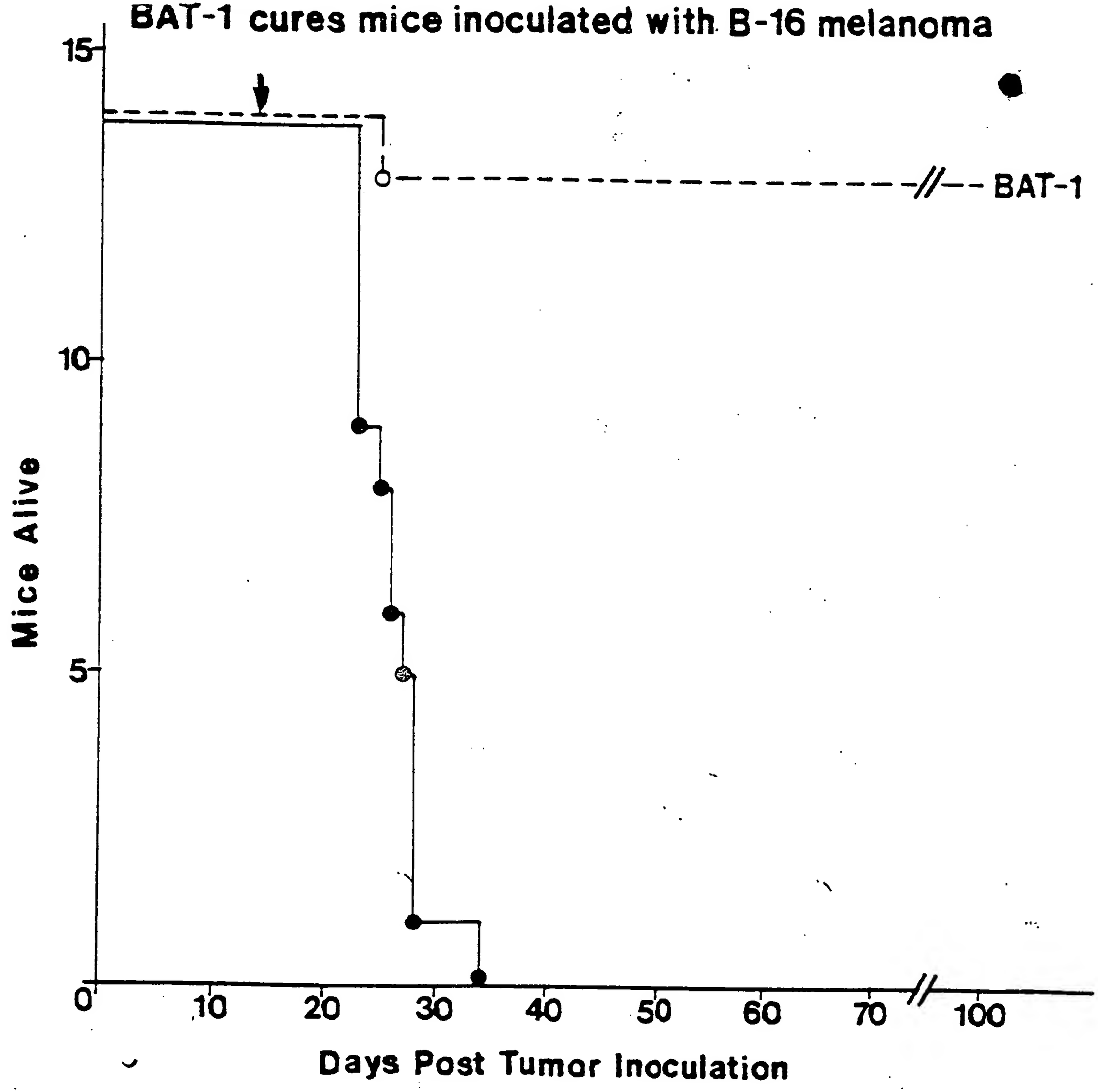

Fig. 10 BAT-1 cures mice inoculated with B-16 melanoma